Antiinflammatory and Antihyperlipidemic Activities of Root Extracts of *Hemidesmus indicus*: *In vitro* and *In vivo* Studies

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Abstract

**Background:** *Hemidesmus indicus* (HI) (Asclepiadaceae), commonly called as Indian Sarsaparilla is traditionally used for blood purification and to treat cardiovascular diseases. The present study is aimed to investigate the (i) anti-inflammatory, (ii) anti-hyperlipidemic, effect of alcoholic and water extracts of HI roots (HIAE and HIWE) using LPS/PMA induced human monocytic (THP-1) cells and high fat diet fed male Wistar rats respectively.

**Methods:** The phytochemical analysis of HIAE and HIWE was done using LC-Q-TOF-MS. Cytotoxicity of the plant extracts was tested by MTT assay. The anti-inflammatory effect of alcoholic and water extracts of HI roots was assayed using lipopolysaccharide (LPS) or phorbol-12-myristate-13-acetate (PMA) induced THP-1 cells. The study was performed by assaying the following markers: tumour necrosis factor alpha (TNF-α) secretion by ELISA, gene expression of proinflammatory markers by q-RT-PCR, translocation of nuclear factor kappaB (NF-κB) by confocal microscopy. Macrophage differentiation markers were also studied by q-RT-PCR. Further anti-hyperlipidemic effect was studied by testing serum lipid profile in high fat diet fed male Wistar rats.

**Results:** Dosage of the plant extracts limited based on cytotoxicity, there was no cell death at 1 mg dwt/mL of HIAE and HIWE. The plant extracts significantly inhibiting the gene expression of LPS induced proinflammatory markers (TNF-α, IL-6 and MIP-1α) and also secretion of TNF-α protein. The extracts also inhibited the translocation of NF-κB-p65 to the nuclei in LPS induced THP-1 cells. Extracts inhibited PMA induced differentiation of monocytes to macrophages, gene transcripts of CD14 and TLR2, however, there was no significant effect on TLR4 transcripts. Further, the study demonstrated anti-hyperlipidemic activity of HI root extracts (at 200 mg/kg body weight/day), treatments not only inhibited high fat diet induced serum lipid markers (total triglycerides, total cholesterol, LDL-cholesterol but also maintained good cholesterol, HDL.

**Conclusion:** The present study for the first time demonstrated anti-inflammatory (*In vitro*) and anti-hyperlipidemic (*In vivo*) effect of HI roots extracts supporting its traditional use for cardioprotective benefits.

**Keywords:** Inflammation; *Hemidesmus indicus*; THP-1 cells; TNF-α; Wistar rats

**Abbreviations**

AE: Alcoholic Extract; HDL-cholesterol; High-Density Lipoprotein Cholesterol; HFD: High Fat Diet; LDL-cholesterol; Low-Density Lipoprotein Cholesterol; PMA: Phorbol-12-Myristate-13-Acetate; TG: Tri Glycerides; TC: Total Cholesterol; WE: Eater Extract

**Background**

*Hemidesmus indicus* (L) R.Br. belongs to family Asclepiadaceae; (phylum: Magnoliophyta; order: Gentianales), commonly named as Indian Sarsaparilla. It has been used in folk medicine and Ayurvedic system of medicine to treat against the diseases of inflammation and other blood related diseases [1-2]. The dried roots of these plants are well known in Indian pharmacopoeia as the drug ‘Anantmul’, a common tonic prescribed for blood purification and other physiological disorders. Several scientific reports have been made on pharmacological benefits of *H. indicus* roots, such as hypoglycemic, antioxidant and cardioprotective properties using animal models [3-5]. Polyherbal formulations containing *H. indicus* (HI) shown to have hypolipidaemic activity using animal models [6-7], it also confirmed protective effect of HI in CCl4 induced alteration of lipid profile in

[1] 1
In view of the role of activated monocytes and macrophages in the pathophysiology of atherosclerosis, the present study was designed to investigate the effect of HI-root extracts (HIAE/HIWE) on LPS and/or PMA induced activation of human monocytic (THP-1) cells and also on lipid profile of high fat diet fed rats. Increased level of lipid markers i.e hyperlipidemia in serum is associated with increased risk of cardiovascular diseases (CVD), atherosclerosis [9]. Atherosclerosis is developed due to complexed interaction between activated blood monocytes and vascular endothelium due to infection or hyperlipidemia, mediated by the release of inflammatory cytokines [9]. High fat diet promotes hyperlipidemia, eventually leads to atherosclerotic plaque formation [10].

Therefore, in the present study, high fat fed rats have been used as experimental models for testing hypolipidaemic effect of HI-root extracts.

### Methods

**Chemicals and reagents**

Power SYBR green PCR master mix was obtained from Applied Biosystems (Carlsbad, CA, USA), BD OptEIA human TNF-α ELISA kit was purchased from BD Biosciences (USA), lipid profile kits were from ERBA diagnostic Mannheim GmbH (Germany), iScript cDNA synthesis kit from Bio-Rad (CA, USA) and nuclear factor kappaB (NF-κB-p65) polycolonal rabbit antibody from Thermo Scientific, (USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Merck, alexa fluor 594 goat anti-rabbit IgG (H+L), fetal bovine serum (FBS), pen-strep, prolonged gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI), RPMI 1640, trizol reagent were purchased from Invitrogen (Germany). Bovine serum albumin (BSA), lipopolysaccharide-<i>Escherichia coli</i> (LPS), paraformaldehyde,
Phorbol-12-myristate-13-acetate (PMA), trypan blue and DEPC treated water were purchased from Sigma-Aldrich (Germany).

**Plant material collection and preparation of HI-root extracts**

The roots of the *H. indicus* (voucher # 46808) were obtained from UoH campus, authenticated by Dr. K. Venkata Ratnam and were deposited in the Department of Botany, Sri Krishnadevaraya University Herbarium (SKU), Anantapur, India. The collected roots were allowed to shade dry for at least 8 to 10 days and stored at room temperature in the dark until further use.

Alcoholic and water extracts from HI roots were prepared [11] using 1 g of dried fine root powder extracts stored until further use at 4°C not exceeding more than 3 days. The alcoholic and water extracts yielded 3.7% (w/w) and 2.9% (w/w) respectively. The effect of respective vehicle concentrations were tested in all sets of cell based experiments.

**ESI-LC-MS/MS analysis**

A mass spectrometry analysis of HIAE and HIWE was conducted using high performance liquid chromatography coupled to 6520 accurate quadrupole time of flight mass spectrometer (HPLC-Q-TOF-MS) (Agilent Santa Clara, CA). Metabolites of the extracts were separated using HPLC reverse phase column. Solvent A and solvent B of mobile phase were ammonium formate (1mM) with formic acid (0.1% v/v) and acetonitrile with formic acid (0.1% v/v) respectively [12]. ESI parameters: both on negative and positive ion mode; mass range 100-1700; spray voltage 4 KV; gas temperature 325ºC; gas flow 10 L/min; Nebulizer 40 psi.

Normal and HFD diet fed male Wistar rats were put on HIAE/HIWE (200 mg/kg/day) for 30 days. At the end of 30 days treatment, lipid markers of serum sample was analyzed by microplate method. Data is expressed as mean ± SD (n= 7 animals). Serum total cholesterol (TC); triglycerides (TG); LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C).

**Table 2: Effect of HI-root extracts on high fat diet induced lipid profile.**

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<tr>
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<th>parameters</th>
<th>HFD (mg/dL)</th>
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<th>HFD (mg/dL)</th>
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<tr>
<td>1</td>
<td>Control group</td>
<td>58.7 ± 3.34 (100%)</td>
<td>108.9 ± 2.13 (185%)</td>
<td>68.1 ± 3.56 (100%)</td>
<td>127.7 ± 9.2 (187%)</td>
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<td>2</td>
<td>HIAE</td>
<td>64.1 ± 3.62 (109%)</td>
<td>78.4 ± 5.19 (134%)</td>
<td>64.2 ± 7.53 (94%)</td>
<td>73.2 ± 6.69 (107%)</td>
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<tr>
<td>3</td>
<td>HIWE</td>
<td>65.6 ± 4.58 (111%)</td>
<td>55.4 ± 6.45 (81%)</td>
<td>54.3 ± 6.47 (80%)</td>
<td>37.8 ± 3.51 (141%)</td>
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Cell viability was examined by MTT assay. Vehicle control was limited to <1%. Experiments were performed at least in triplicate and the results were expressed as the mean ± S.D, n=6.

**Figure 1: Cytotoxic effect of HI root extracts on human monocytic (THP-1) cells.**

The cells were incubated with different concentrations of HIAE and HIWE for 24 h at 37°C and 5% CO₂. Media without cells but with respective concentrations of plant extracts were used as appropriate blanks and the cell viability was examined by MTT assay. Vehicle control was limited to <1%. Experiments were performed at least in triplicate and the results were expressed as the mean ± S.D, n=6.

**Figure 2: Effect of HI-root extracts on proinflammatory markers of LPS induced THP-1cells.**

A) Effect of HI-root extracts (1 mg dwt/mL) on gene expression of IL-6; TNF-α and MIP-1α in control vs LPS induced THP-1 cells. B) Effect of HI root extracts on LPS-mediated TNF-α secretion in THP-1 cells. Cells were seeded at 5x10⁵ cells/mL. Data represents fold change in TNF-α release into the media by the cells treated with LPS (0.5 µg/mL for 3h) compared to untreated cells. HIAE and HIWE added to the cells 12h prior to incubation with LPS. Vehicle volume (alcohol or water) did not exceed 1% and had no effect. Experiments were performed at least in triplicate and the results are expressed as the mean ± S.D. *p<0.001 for comparison between Cells+LPS vs cells-LPS. ^p<0.001 compared between cells treated with LPS in the presence of plant extracts vs in their absence.

**Cell culture and treatments**

The human monocytic (THP-1) cells were obtained from National Centre for Cell Sciences, India. The cells were cultured at 37°C in 5% CO₂ in RPMI media supplemented with 10% fetal bovine serum (FBS).

Cell viability with various concentrations of plant extracts was assayed using MTT reagent [11]. All the experiments were performed at a cell density of 5 x10⁴ cells/mL. An acute inflammatory stimulus was given to the cells by incubating with 0.5 µg/mL of LPS for 3h at 37°C in 5% CO₂. In order to stimulate the differentiation of THP-1 cells to macrophages, cells were treated with PMA (5 µg/mL) or MIP-1α for 48 h at 37°C in 5% CO₂ [13]. The images of the cells were taken under inverted microscope.

**Transcript analysis**

After respective treatments with LPS or PMA, in the presence or absence of plant extracts, total RNA from the cells was isolated using trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions. Subsequently, one microgram of RNA was used for c-DNA synthesis (Bio-Rad synthesis kits). Then the transcripts were quantitated by real time PCR using specific set of primers.

**Quantification of TNF-α**

Cells were incubated with or without plant extracts (1 mg dwt/mL) at 37°C for 12h. After LPS treatment, cells were centrifuged and
collected supernatants were stored at -80 ºC until further use. TNF-α present in the supernatants was determined using TNF-α ELISA kit as per the manufacturer’s protocol.

**Nuclear translocation of nuclear factor kappaB (NF-κB)**

The NF-kB-p65 in THP-1 cells was detected by indirect immunofluorescence assay using confocal microscopy [14]. After the respective treatments of plant extracts, the cells were fixed (4% paraformaldehyde) and blocked with 3% BSA. Subsequently polyclonal antibodies against NF-kB-p65 (1:250) were applied for 12h (4% paraformaldehyde) and blocked with 3% BSA. Subsequently the respective treatments of plant extracts, the cells were fixed

Toxicity of plant extracts, if any, on THP-1 cells was determined by Toxicity of plant extracts, if any, on THP-1 cells was determined by Toxicity of plant extracts, if any, on THP-1 cells was determined by Toxicity of plant extracts, if any, on THP-1 cells was determined by toxicity assay using MTT as shown in (Figure 1). No cell death was observed up to 1 mg dwt/mL of HIAE and HIWE. Vehicle solvents (alcohol or water of <1%) had no effect on cell viability.

**Effect of HIAE and HIWE on LPS induced proinflammatory gene expression**

The effect of the plant extracts on LPS induced gene expression of inflammatory markers was tested at transcriptional level. The transcript analysis by real-time quantitative PCR showed that LPS stimulated significantly the expression of IL-6 (2 fold), TNF-α (4.7 fold) and macrophage inflammatory protein (MIP-1α) (2 fold) as shown in (Figure 2A). Such stimulation of proinflammatory gene expression was markedly inhibited in the cells pretreated with HIAE/ HIWE (1 mg dwt/mL).

**Effect of HIAE and HIWE on the subcellular localization of NF-κB-p65**

The effect of HIAE and HIWE on the subcellular localization of NF-κB-p65 was investigated. The transcription factor NF-κB plays a crucial role in the regulation of gene expression of several proinflammatory markers including TNF-α. Results presented in the (Figure 3) clearly indicated that

**Experimental design of animals**

Male Wistar rats of 126 ± 9.3 g average body weight were obtained from National Institute of Nutrition, Hyderabad and used throughout the study. The animals were maintained in the animal house on University’s campus as per the CPCSE guidelines and regulation. The animals were kept in individual cages and allowed to acclimatize for a week. The study was approved by the Institutional Animal Ethics Committee Institutional Animal Ethics Committee approved as per the guidelines of Committee for the Purpose of Control and Supervision on Experiments, New Delhi, India (IAEC/ CPCSEA, LS/IAEC/SDT/11/02). Food and water were supplied ad libitum continuously. Hyperlipidemia was induced feeding high fat diet (HFD) of Amul butter 2 g/day for 30 days. Rats were randomly assigned to six groups of 7 animals each. Group I: normal diet (for 30 days), group II: HFD, group III: HIAE (200 mg/kg body weight/day), group IV: HIAE (200 mg/kg body weight/day) + HFD, group V: HIWE (200 mg/kg body weight/day) and group VI: HIWE (200 mg/kg body weight/day) + HFD. At the end of 30 days, the rats were fasted overnight and sacrificed. Blood sample was obtained by cardiac puncture of anaesthetized rats. Serum was separated from the blood and subsequently analyzed for total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol using commercially available kits (Erba Diagnostics Germany). Weight of the rats was recorded before and after respective treatment periods.

**Statistical analysis**

To analyze the obtained data, Sigma Plot 11 software was used. All the data obtained was analysed by one way analysis of variance (ANOVA) test using statistical package for the Life Sciences (SPLS version 11). Data obtained from all the experiments was expressed as mean ± S.D of three individual experiments. P-values less than 0.001 were considered as statistically significant.

**Results**

**Metabolite profiling of HIAE and HIWE by LC-MS/MS**

In the present study, LC-Q-TOFMS was employed for metabolite fingerprinting of the plant extracts. Negative ion and positive ion modes of LC-QTOF-MS detected 7 and 12 compounds in HIAE; 4 and 7 compounds in HIWE respectively. Their abundance and RT values in minutes are shown in (Table 1). The detected compounds belong to pregnane glycoside, terpenoid or coumarinolignoid. Chlorogenic acid, lupeol acetate, hexatriacontane, nerolidol, hemidesmin 1, hemidesmin 2, hemidesminine, β-sitosterol were commonly detected in both HIAE and HIWE. 2-hydroxy-4-methoxy benzoic acid, emidine, lunapone, heminine, desinine, rutin, hemidine were exclusively detected in HIAE, while 4-terpinoine was detected only in HIWE.

**Figure 3: Effect of HIAE and HIWE on NF-κB-p65 in LPS induced THP-1 cells.**

Confocal microscopic images demonstrating the effect of HIAE and HIWE on NF-κB-p65 in THP-1 cells. Images with vehicle control 0.5% (alcohol) cells are shown in panel 1. Vehicle control of water also had no effect (not shown). NF-kB translocation was analyzed by staining with NF-kB-p65, red (A); nucleus with DAPI, blue (B); merged image of NF-kB-p65 and DAPI (C). THP-1 cells treated with LPS (0.5 µg/mL for 3h) shown in 2nd panel (D to F); cells pretreated with HIAE and HIWE (1 mg dwt/mL) and then treated with LPS shown in 3rd (G to I) and 4th panels (J to L) respectively.

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| DAPI | NF-κB/p65 | Merge | THP-1 cells | + LPS | HIAE + LPS | HIWE + LPS

**Effect of HIWE on the subcellular localization of NF-κB-p65**

In order to further elucidate, the molecular mechanism by which the plant extracts modulate gene expression in THP-1 cells, the effect of HIAE/HIWE on transcriptional factor NF-kB was investigated. The transcription factor NF-kB plays a crucial role in the regulation of gene expression of several proinflammatory markers including TNF-α. Results presented in the (Figure 3) clearly indicated that...
The plant extracts inhibited LPS induced translocation of NF-κB in THP-1 cells. The confocal images of Figures 3A-3C (1st panel) showed that NF-κB-p65 was mostly sequestered in the cytoplasm of normal uninduced cells, whereas it was predominantly located in the nuclei of the LPS-stimulated cells (Figures 3D-3F). Such LPS dependent nuclear translocation of NF-κB-p65 was blocked in the cells pretreated with HIAE (Figures 3G-3I) and HIWE (Figures 3J-3L). These results demonstrated that HIAE and HIWE elicited anti-inflammatory activity in THP-1 cells by modulating subcellular localization of transcriptional factor NF-κB.

**Effect of HIAE and HIWE on PMA induced macrophage differentiated markers**

Human monocyte (THP-1) cells are suspension in nature and do not adhere to the plastic surfaces of the culture plates as shown in (Figure 4A(i)). Treatment with PMA (5 ng/mL) stimulated the differentiation of THP-1 cells into macrophages as indicated by their adherence to substratum of the culture dish (Figure 4A(ii)). The adherent cells showed morphological characteristics similar to macrophages. The effect of alcoholic and water extracts of HI roots on such differentiation process was tested by treating the cells with PMA in the presence or absence of plant extracts. Pretreatment with HIAE or HIWE (1 mg dwt/mL) significantly attenuated the process of differentiation in PMA challenged THP-1 cells as shown in (Figures 4A(iii,iv)).

Literature survey clearly demonstrates that differentiated monocytic cells compared to mononcytic cells had an increased expression of CD14, TLR2 and TLR4 which are macrophage-specific antigens. Therefore PMA differentiated THP-1 cells were examined by quantitative real-time PCR to check the expression of selected genes CD14, TLR2 and TLR4 compared to untreated cells on the basis of fold changes. Results showed that PMA upregulated the expression of CD14 by 3 fold and toll like receptors (TLR2 and TLR4) by 64 and 37 fold respectively (Figure 4B). Such upregulation of the markers was markedly attenuated by HIAE/HIWE (1 mg dwt/mL). However, plant extract did not show much effect on TLR4 expression (Figure 4B).

**Effect of HIAE and HIWE on HFD induced serum lipid profile in rats**

Administration of HFD in rats shows significant increase in their serum lipid markers like total cholesterol:108 mg/dL(185%); triglyceride: 127 mg/dL (187%); and LDL-C: 64 mg/dL (240%) levels with respect to the baseline value of normal rats (Table 2) whereas serum HDL-cholesterol level has reduced to 53 mg/dL(77%). In the present study, co-treatment with HIAE or HIWE prevented such HFD induced increase in serum total cholesterol, LDL-cholesterol, triglycerides and decrease in HDL-cholesterol as shown in (Table 2).

**Discussion**

In recent years, increasing interest has been recognized in developing and developed countries for use of herbal products for maintaining primary health care [15]. Polyherbal drug containing HI-roots namely PHF-AROGH and RO7D are shown for their analgesic and antiulcer properties in animal models [16-17]. However, mechanism of action of HI-roots in offering anti-inflammatory property and its relevance to cardiac health remains unclear. To the best of our knowledge, this is the first attempt that has been made to understand the biochemical basis for anti-inflammatory and anti-hyperlipidemic activity using human cell culture and animal model.

In this study, acute inflammatory model i.e. LPS/PMA induced THP-1 cells were used to evaluate anti-inflammatory activity of HI-roots. TNF-α is a potent proinflammatory cytokine secreted by monocytes in the blood and plays a major role in developing many inflammatory diseases [18]. The markers studied IL-6 and MIP-1α play a significant role in inflammation associated with atherosclerotic lesion development and LPS activates the expression of selected markers via nuclear translocation of NF-κB pathway [19]. HI-root extracts significantly inhibited gene expression of above markers and also nuclear translocation of NF-κB in LPS stimulated THP-1 cells. These results suggest that the plant extracts regulate gene expression by modulating subcellular localization of NF-κB. Studies in *Apoe−/−/ Myd88−/−* double knockout mice demonstrated that CD14, TLR2 and TLR4 are strongly associated with inflammation and atherosclerosis development [20]. The extracts inhibited the expression of PMA induced cell differentiation markers CD14 and TLR2 as well as...
morphological characteristics of macrophages.

Dietary cholesterol used as composition of HFD causes increase in serum cholesterol and decrease in HDL-cholesterol and it has been widely known that elevation of serum cholesterol and triglyceride can lead to atherosclerosis and coronary heart diseases and High levels of LDL cholesterol are deposited in the interior of blood vessels resulting in hardening of arteries and narrowing of the blood vessels [21]. There are few reports published on hypolipidaemic activity of polyherbal formulations containing H. indicus (HI) [6-8] or its callus cell culture extract using animal models [22]. In the present study, co-treatment of HI-root extracts significantly reduced serum lipid level in high fat diet fed rat model demonstrating hypolipidaemic activity of phytoconstituents of HI root extracts.

Furthermore, LC-MS analysis showed the presence of several bioactive secondary metabolites in HIAE and HIWE. Compounds namely 2-hydroxy-4-methoxy benzoic acid, β-sitosterol shown for their pharmacological activities [23,24]. Chlorogenic acid (37 µg/mL) showed for their LPS induced anti-inflammatory property in RAW264.7 cells [24]. In summary, the present study validated traditional use of HI roots for cardioprotective effect using THP-1 cells and Wistar rats and also showed several bioactive compounds can be extracted using solvents like alcohol and water extracts. H. indicus which can be grown widely in tropical and subtropical regions of the world, therefore, HI-roots can be considered as potential resource drug candidates for treating inflammatory diseases like atherosclerosis.

**Conclusion**

The present study highlights the effectiveness of HI root extracts in attenuating the proinflammatory and hyperlipidemic response. Critical cellular and molecular investigation of the present study demonstrates that alcoholic and water extracts of HI roots: (i) Had bioactive compounds known to have anti-inflammatory properties like pregnane glycoside, terpenoid, coumarinolignoid (ii) Can attenuate LPS induced activation of proinflammatory gene expression in THP-1 cells by inhibiting nuclear translocation of NF-kB; (iii) Can inhibit PMA induced monocyte to macrophage differentiation; (iv) Can significantly reduced the HFD fed hyperlipidemia in animal model. Thus the present study supports the anti-inflammatory and anti-hyperlipidemic activity of HI-roots. Further studies using in vivo models like Apoe-/- atherosclerotic mice would help in validating use of H. indicus roots in treating cardiovascular diseases.

**Author’s Contribution**

SSC involved in performing experiments, data analysis and writing the manuscript. SDT involved in inception of the project, raising funds required to perform the experiments, designing and directing of all the experiments, correction and evaluation of entire manuscript.

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